LYSINE 216 IS A BINDING SITE OF THE RETINYL MOIETY IN BACTERIORHODOPSIN

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1. Introduction

Bacteriorhodopsin (bR) in the purple membrane (PM) of *Halobacterium halobium* acts as a light-driven proton pump and contains retinal in a stoichiometry of 1:1. Resonance Raman spectra revealed a C=N double bond between retinal and the protein in the native chromophore [1,2]. This Schiff base is not reducible in the dark but a retinyl moiety is produced by the combined action of light and sodium borohydride [3]. Alkaline hydrolysis of this photoreduced membrane, called RP_{hv} yielded a retinylamino acid, which was identified as retinyllysine by mass spectroscopy [4].

In [5], [^{14}C]succinylation after delipidation of the purple membrane or RP_{hv} was used for specific detection of lysine containing peptides. One of these peptides (called A in [5]) was reported to have the sequence Val–Ser–Asp–Pro–Asp–Lys–Lys carrying 2 succinyl residues. As a consequence of reduction of the membrane, peptide A was missing in the fingerprint but a new peptide with a different mobility (called F in [5]) was obtained and subjected to automatic Edman-degradation. It released radioactivity in cycle 6. The authors concluded that peptides A and F were the same but that in peptide F the C-terminal lysine carries the retinyl moiety, and hence cannot be succinylated. This explains the difference in mobility. Based on these results it was suggested [6] that the peptide sequence of A and F coincide 'basically' with positions 34–41 of the protein and that lysine 41 is the retinal binding site. However, an additional alanine between aspartic acid and lysine was also reported [6] and confirmed in [7]. Therefore the correct sequence of the peptide segment carrying positions 34–41 is Val_{34}–Ser–Asp–Pro–Asp–Ala–Lys–Lys_{41}. Obviously, no radioactivity can be released in sequencing step 6 from this peptide, because alanine can not react with [^{14}C]succinic anhydride. Therefore, no evidence remains that lysine 41 is the retinal binding site in RP_{hv} or bR. This paper describes experiments which show that the retinyl residue in RP_{hv} is bound to lysine 216 (numeration as in [7]).

Abbreviations: bR, bacteriorhodopsin; PM, purple membrane; RP_{hv}, borohydride reduction product of purple membrane in the light; HPLC, high performance liquid chromatography; SDS–PAGE, sodium dodecylsulphate polyacrylamide gel electrophoresis; PTH, phenylthiohydantoin

2. Materials and methods

Halobacterium halobium strain S9 was grown and purple membrane isolated as in [8]. Reduction with 0.6% $NaBH_4$ was carried out as in [4] or at 2°C for various times depending on the amount of bR (bR was held at 16 μM). Chymotryptic cleavage (chymotrypsin from Boehringer) was done as in [9] for 5 h but with 100 μM protein and a ratio of enzyme to protein of 1:4. Delipidation, subtilisin cleavage and amino acid analysis were as in [10]. Synthesis of retinyllysine followed [11] starting with *N*-α-benzoxycarbonyllysine. A molar absorbancy of 52 $mM^{-1} \cdot cm^{-1}$ was used for quantitation of retinyl compounds [12]. Peptides were separated on an isocratic HPLC system on Merck Si 60 material (7 μm, column size 4.6 × 250 mm, Gynkothek pump, 600/200, Kontron Uvikon 725 flow photometer) using $CHCl_3/CH_3OH/NH_3$ (70:27:3) as solvent. The C-terminus of the retinyl peptide (52 nmol) was coupled to aminopolystyrene (30 mg) for 1.5 h at 30°C with a yield of 88% (45 nmol) [13] and the excess of resin amino groups was blocked with phenylisothiocyanate. The peptide was sequenced manually with phenylisothiocyanate following the

suggestions in [14]. At cycle 3, 1 N HCl in CH_3OH at 50°C for 10 min was used for recovery of PTH-serine [15]. Identification of PTH-amino acids was as in [16] but different support material (Shandon ODS, 3 μm, Bischoff) was used and the flow rate raised to 2.5 ml/min (resulting in a back pressure of 260 bars at 62°C).

For SDS–PAGE a linear polyacrylamide gradient (9–24%) in the system of [17] was employed.

3. Results and discussion

Reduction of bR in the light at 2°C for 15 min yields a retinyl protein which fluoresces strongly due to its retinyl moiety and migrates as a single band on SDS–PAGE as shown in fig.1(4,6).

Subsequent chymotrypsin treatment cleaves the protein between residues 71 and 72 as reported in [6] producing fragments around M_r 15 000 and M_r 8000. Sequence analysis of bR after chymotryptic treatment gives exclusively Gly_{72}–Gly_{73}–Glu_{74}–Gln_{75} as expected for bR as an N-terminally blocked polypeptide.

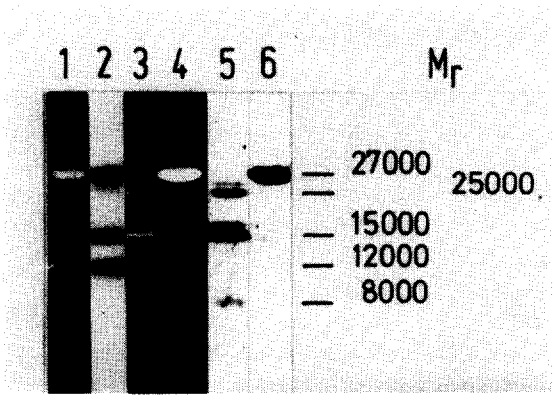


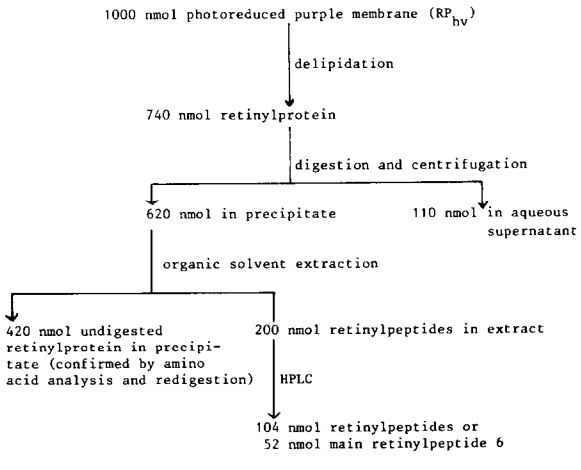
Fig.1. SDS–PAGE of RP_{hv} and its degradation products: (1,2) PM was reduced as in [4] but at 30°C. After bleaching incubation was continued in the dark overnight; (4,6) PM were reduced as in [4] but at 2°C until the sample was bleached (for 10 nmol PM this occurs within 15 min); (3,5) chymotrypsin-digested PM, photoreduced as sample 4 and 6. Photographs of 1, 3 and 4 were taken from unstained gels under illumination with an UV-lamp. The camera was protected under a light yellow filter. Photographs 2, 5 and 6 were taken from the same gels after Coomassie blue staining. M_r-values for intact bR (27 000), fragment 72–239 (17 500) and fragment 1–71 (800) were used for calibration of other bands in a semilogarithmic plot.

Fig.1(5) shows Coomassie blue staining of chymotrypsin digested, photoreduced bR. The 2 bands with only slightly reduced molecular masses (~M_r 25 000) compared to bR (M_r 27 000) arise from carboxyterminal digestion by chymotrypsin at two different sites (see also [6] and [18]). For the same reason the C-terminal fragment has 2 bands at ~M_r 15 000. Although the fragments M_r 15 000 and M_r 8000 must be present in a ratio of 1, the Coomassie stain apparently is less intense in the small fragment (fig.1(5).

The fluorescence distribution of the chymotrypsin digested photoreduced bR is shown in fig.1(3). The ~15 000 M_r carboxy-terminal fragments are highly fluorescent, while the small 8000 M_r fragment carrying residues 1–71 has only a trace of artefactual (see below) fluorescence. Thus the retinyl binding site is located at a lysine residue beyond position 71 and lysine residues at positions 30, 40 and 41 are excluded.

Lysine 129 was excluded as the retinyl binding site on the basis of experiments where bR was specifically cleaved by borohydride in a side reaction. Prolonged incubation with borohydride at 30°C results in the production of two fragments of unequal size (M_r 12 000 and 15 000) as shown in fig.1(2) by staining with Coomassie blue. The 12 000 M_r fragment carries the retinyl binding site (i.e., is fluorescent, fig.1(1)). This fragment must be the C-terminal fragment since chymotryptic digestion (see above) produces larger 15 000 M_r C-terminal fragments which carry the retinyl binding site. Thus the retinyl binding site is located

Scheme 1



Isolation of retinylpeptides

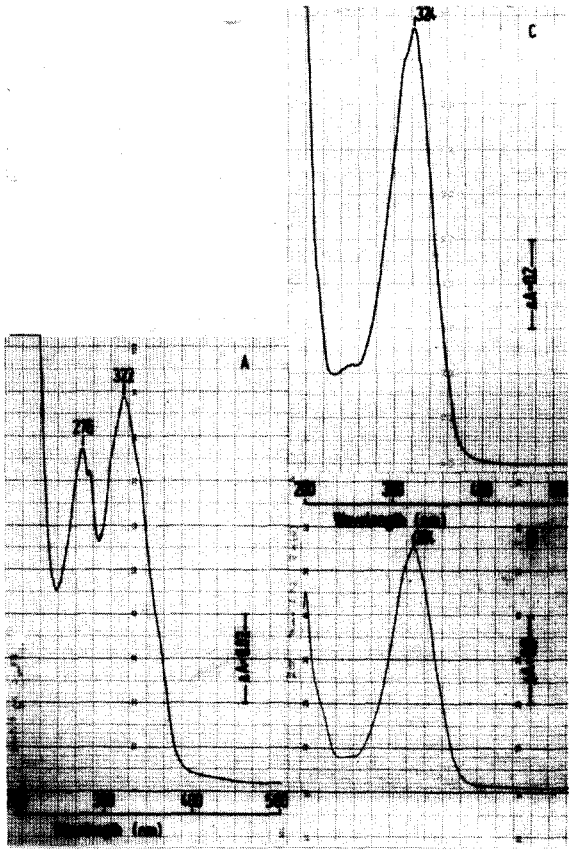


Fig.2. (A) UV-spectrum of peptides in the ethanol extract of subtilisin cleaved photoreduced bR. (B) UV-spectrum of the main retinyl peptide (fig.3(6)) in ethanol after HPLC. A shoulder at 220 nm may be due to peptide bond absorption. (C) UV-spectrum of synthetic *N*-ε-retinyl lysine in ethanol.

beyond residue 138 (12 000 M_r from the C-terminus) or conversely is not found in residues 1–137 because the 15 000 M_r N-terminal fragment in the borohydride reaction is not fluorescently labelled (fig.1(1)).

The trace of fluorescence observed at the band around 8000 M_r after chymotrypsin treatment can be explained by carboxy-terminal digestion of the small amounts of the 12 000 M_r fragment produced by borohydride treatment even at 2°C (see fig.1(1)). This explains the observation of varying amounts of retinyl label on small and large fragments under various conditions of reduction and chymotryptic cleavage [19].

Further experiments had to define the exact position of the lysine carrying the retinal moiety in photoreduced bR. The protein was cleaved with subtilisin and the retinyl peptides were isolated according to scheme 1. Water soluble peptides were removed by centrifugation and hydrophobic peptides extracted from the sediment with organic solvents. The ratio of the absorbance at 276 and 322 nm in the organic extract (fig.2A) was very low compared to that of the aqueous supernatant indicating enrichment of retinyl compounds. Upon a second purification step using HPLC (fig.3) one main peak absorbing at 324 nm was obtained (fig.2B), which was spectroscopically indistinguishable from retinyllysine (fig.2C). The amino acid composition of this material yielded: Asp (0.94), Ser (0.79), Gly (0.43), Val (0.89), Leu (0.46) and Lys (0.12) and a background (~0.1) of most of the other amino acids when compared with Ala (1.00) suggesting a peptide Asp, Val, Ser, Ala, Lys carrying a retinyl moiety and being contaminated mainly by Gly and Leu. Lysine was not expected to be found in stoichiometric amounts because of retinyllysine destruction during acid hydrolysis [20]. The minor component 5 in the chromatogram had an amino acid composition similar to the main compound (fig.3, peak 6) and may represent a *cis/trans* isomer of the retinyl moiety in the same peptide. Peaks 1–4 contained only amino acids also found in peak 5 and 6 but some amino acids were missing. When the aqueous supernatant of the subtilisin digest was subjected to HPLC the same retinyl peptides but in a different ratio were found, peptide 6 being a minor component. Varying conditions of subtilisin digestion also changed the relative amounts of the various peptides. We therefore assume that these peaks (1–4) contain smaller peptides than peak 6 but they were of the same origin in the sequence of bR.

These results suggested lysine 216 as a retinyl binding site in RP_{hv} and this was confirmed by sequence analysis of peptide 6. After covalent linkage of the peptide to aminopolystyrene it was degraded manually by the Edman-procedure and the PTH-amino acid derivatives were analysed by HPLC. In fig.4 a standard mixture (A), the product from the cycle 2 (B) and the blank after the step 5 (C) are compared.

Table 1 summarizes the results and establishes the sequence Asp–Val–Ser–Ala which are the positions 212–215 of the polypeptide chain. This sequence precedes retinyllysine 216 of the peptide linked to the resin with its C-terminus. Arg_{134}–Phe_{135}–Val_{136} was found as a contaminant. The main contaminants Gly and Leu found in amino acid analysis did not

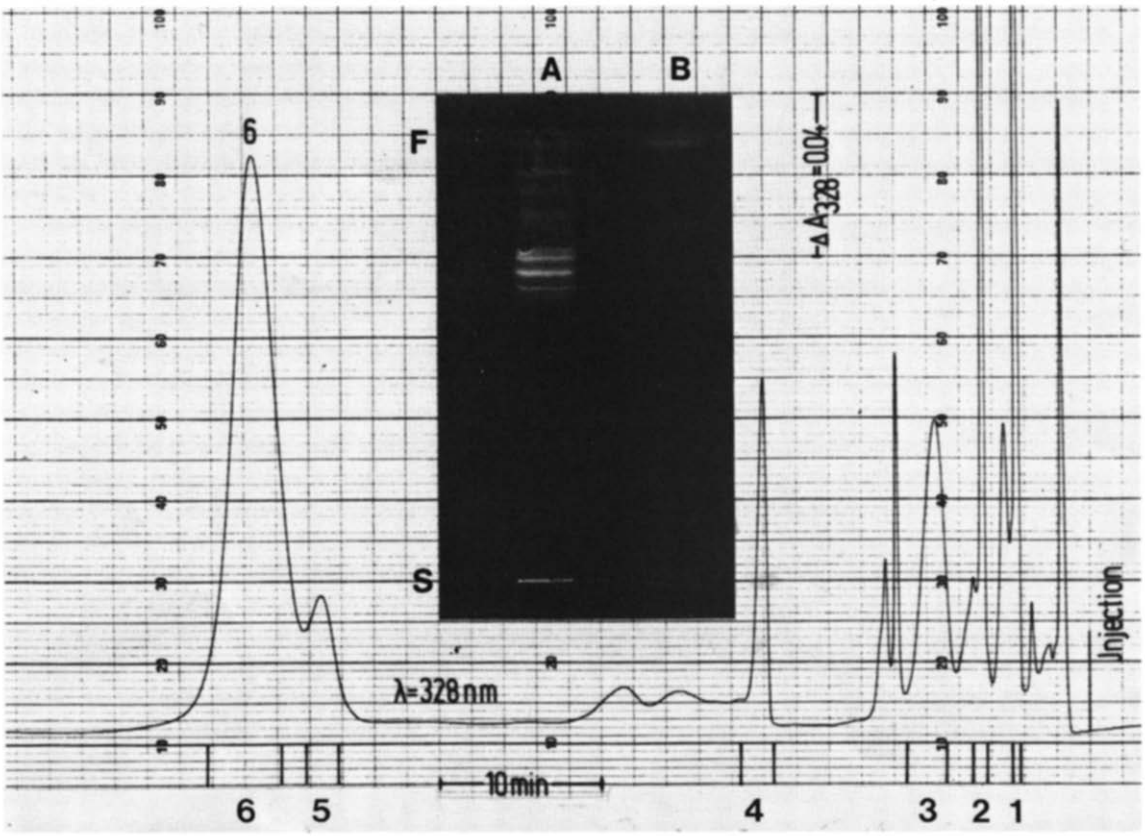


Fig.3. HPLC of ethanol-soluble peptides (see spectrum in fig.2) after subtilisin cleavage of photoreduced bR. Retinylpeptides were detected at 329 nm. The inset shows a thin-layer chromatogram (Silica gel plates Merck, same solvent as in the HPLC experiment). For detection the chromatogram was photographed under a UV-lamp as described for the gels to establish the presence of fluorescing substances (A) migrating slower, i.e., being more polar than retinol (B).

show up in sequence analysis. This could be due to different coupling yields of the various contaminating peptides. No explanation can be given for Tyr as a contaminant in cycle 4. The peptide's sequence unambiguously establishes lysine 216 as a binding site of the retinyl moiety in RP_{hv}. Quantitative analysis of the yield of peptide 6 shown in the scheme below indicates that lysine 216 must be the main binding site. The sequenced peptide 6 amounts to 50% of the organic solvent extracted material, the minor components are very likely derivatives of this peptide and the aqueous supernatant contains the same retinyl peptide mixture as the organic solvent extracted material.

Thus, lysine 41 is not the binding site, but our new information demonstrating that it is lysine 216 does not substantially alter the location of the retinyl moiety in a proposed structural model of bR [21] since lysine 216 is located in a similar position as lysine 41.

Analysis of the retinyl binding site requires reduction of the retinylidene moiety in a light-dependent reaction not well understood. Therefore no conclusion about the retinal binding in native purple membrane can be derived with certainty although the retinyl moiety blocks the regeneration reaction of RP_{hv} with retinal, indicating that the retinyl residue occupies the retinal binding cavity [3].

Additionally, we found that dark reduction of bacteriorhodopsin in the non-crystalline state yields the same retinyl peptide mixture as RP_{hv}, indicating that lysine 216 indeed is also a binding site for retinal in native bacteriorhodopsin.

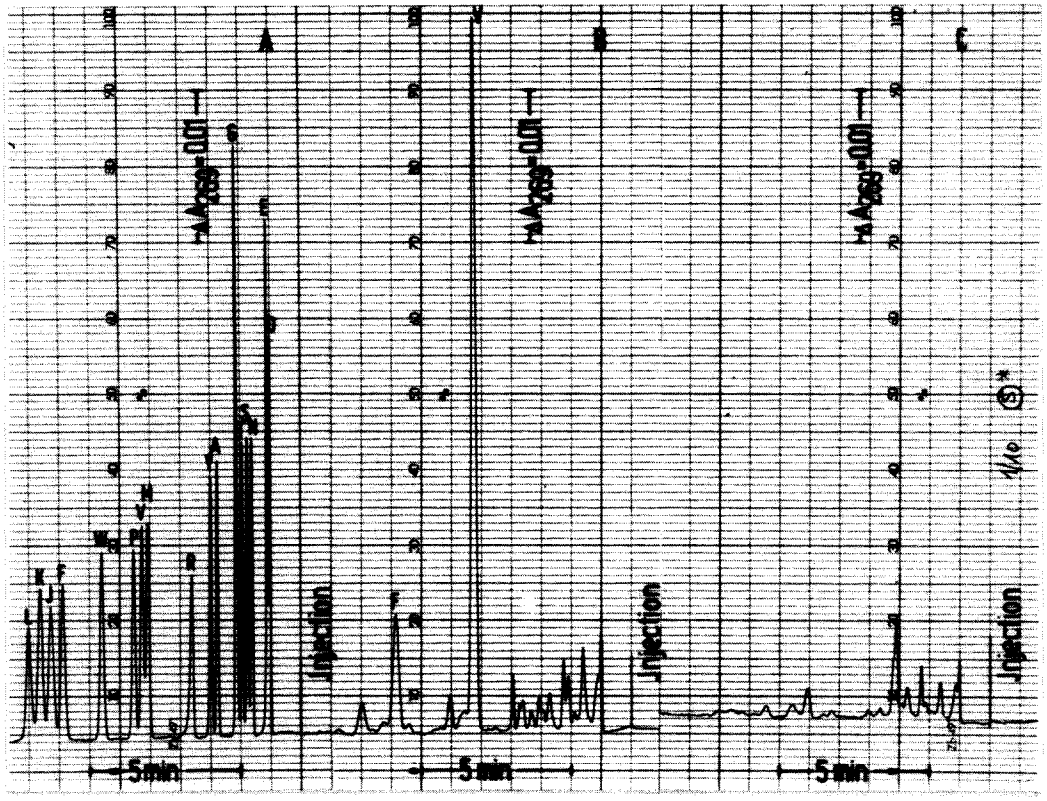


Fig.4. (A) Separation of PTH-amino acids (500 pmol/component) on reversed phase HPLC as in section 2. (B) Second cycle of the manual solid phase sequencing of the retinylpeptide 6 of fig.2. PTH-valine is the main compound released, whereas PTH-arginine is present as an impurity. (C) Cycle 5 (blank) of the same experiment.

Table 1
Solid phase Edman degradation of 45 nmol retinylpeptide 6

PTH derivative of	Retention time (in minutes) in				
	Standard	Cycle 1	Cycle 2	Cycle 3	Cycle 4
Asp	1.04 (0.5)	1.04 (8.0)			
Ser	1.80 (0.5)			1.75 (4.0)	
Ala	2.65 (0.5)				2.63 (9.0)
Tyr	3.04 (0.5)				3.02 (3.3)
Arg	3.65 (0.5)	3.60 (4.0)			
Val	5.35 (0.5)		5.27 (22.0)	5.26 (5.7)	
Phe	7.99 (0.5)		7.87 (5.2)		

It was carried out as described in [22]. The results of PTH-amino acid analysis by HPLC were quantitated by an Shimadzu integrator E 1A. Values in brackets are nmol PTH-amino acid calibrated with a standard mixture. For proof of identity the retention times (figures without brackets) of the standard PTH-amino acids and the main components found in each cycle are given. The low yield of aspartic acid in the first step is explained by a side reaction in which additionally its carbonyl group is coupled to the resin. Therefore, the 22 nmol PTH-Val in cycle 2 was used to measure the yield of the material which was sequenced off the resin (50% as compared to the amount of material attached). This compares well with [23]. The low yield of PTH-serine in cycle 3 is explained by destruction during degradation [15]

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